AD_____

Award Number: DAMD17-01-1-0353

TITLE: Breast Cancer Prevention by a Fatty Acid Binding Protein
MRG-Induced Pregnancy Like Mammary Gland Differentiation

PRINCIPAL INVESTIGATOR: Mingsheng Wang, Ph.D.
Y. Eric Shi, M.D., Ph.D.

CONTRACTING ORGANIZATION: Long Island Jewish Medical Center
Lake Success, New York 11042

REPORT DATE: August 2002

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are
those of the author(s) and should not be construed as an official
Department of the Army position, policy or decision unless so
designated by other documentation.

20030313 115

REPORT DOCUMENTATION PAGE

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE August 2002	3. REPORT TYPE AND DATES COVERED Annual Summary (1 Aug 01 - 31 Jul 02)	

4. TITLE AND SUBTITLE
Breast Cancer Prevention by a Fatty Acid Binding Protein MRG-Induced Pregnancy Like Mammary Gland Differentiation

5. FUNDING NUMBERS
DAMD17-01-1-0353

6. AUTHOR(S)
Mingsheng Wang, Ph.D.
Y. Eric Shi, M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Long Island Jewish Medical Center
Lake Success, New York 11042

E-Mail: wang@lij.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract *(Maximum 200 Words) (abstract should contain no proprietary or confidential information)*

A mammary derived growth inhibitor related gene (MRG) was previously identified and characterized. MRG induces differentiation of mammary epithelial cells *in vitro* and its expression is associated with mammary differentiation. Overexpression of MRG in human breast cancer cells induced differentiation with changes in cellular morphology and a significant increase in the production of lipid droplets. Treatment of mouse mammary gland in organ culture with MRG protein resulted in a differentiated morphology and stimulation of β-casein expression. To further define the role of MRG on mammary differentiation, a MRG transgenic mice model under the control of MMTV promoter was established and investigated. While there was no lobulo-alveolar structure in control virgin mice, expression of MRG transgene in the mammary gland resulted in the formation of alveolar-like structure. Consistent with the morphological change, expression of MRG also increased milk protein β-casein expression in the gland. Our results suggest that MRG is a candidate mediator of the differentiating effect of pregnancy on breast epithelial cells.

14. SUBJECT TERMS breast cancer			15. NUMBER OF PAGES 9
			16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited

Table of Contents

A. INTRODUCTION

A-1. M̲ammary derived growth inhibitor (MDGI) R̲elated G̲ene MRG. Mammary gland development is controlled by systemic hormones and by local growth factors that might complement or mediate hormonal actions. In an effort to search growth regulators in the human mammary gland, we generated cDNA libraries from a breast cancer biopsy specimen and a normal breast and analyzed these libraries by differential cDNA sequencing (1). We identified, cloned, and characterized a novel tumor growth inhibitor and named it the M̲ammary derived growth inhibitor-R̲elated G̲ene MRG (2). The predicted amino acid sequence of MRG has a significant sequence homology to previously identified mouse mammary derived growth inhibitor **MDGI** (3). MDGI is a mammary epithelial cell growth inhibitor and differentiation factor initially identified and purified from Ehrlich ascites mammary carcinoma cells (3), and then from the lactating bovine mammary gland (4-5) and from cows milk (6). Studies of **mouse and bovine** MDGI suggest several functions of MDGI on growth and differentiation of mammary gland. MDGI specifically inhibit the growth of normal mouse mammary epithelial cells (MEC), and promote morphological differentiation: the appearance of bulbous alveolar end buds and formation of fully developed lobuloalveolar structures (7). Selective inhibition of endogenous MDGI expression in mouse MEC by use of antisense oligonucleotides suppresses alveolar budding and impairs ß-casein synthesis in organ cultures (7). Increasing amounts of MDGI mRNA were detected in terminal parts of ducts and lobuloalveolar epithelial cells of differentiated glands and maximally expressed in the terminally differentiated state found just prior to lactation (8). MDGI expression in mouse mammary epithelium cells is hormonally regulated (9-10). Many of these growth inhibition and differentiating effects of MDGI are **conserved** in MRG.

A-2. Fatty acid binding protein (FABP). Interestingly, MRG and MDGI revealed no homology to any other known growth inhibitors; rather, they revealed extensive sequence homology to FABP (11-12). A striking homology was evident between bovine MDGI and Heart type (H-) FABP, which differ only in seven positions of the amino acid sequence (13). In fact, it turned out that the originally described MDGI is a mix of H-FABP and adipocyte type (A-) FABP both expressed in mammary gland (14-15). H-FABP fully replaced the MDGI effect and inhibited the growth of mammary epithelial cells (14). Like MDGI and H-FABP, the sequence of MRG was found to be identical to the recently deposited sequences of human brain type **(B-) FABP** in GenBank (accession #AJ002962) (12). Cellular FABPs are a highly conserved family of proteins consisting of several subtypes and have been suggested to be involved in intracellular fatty acid metabolism and trafficking. Among them, only H-FABP/MDGI and the recently identified B-FABP/MRG have a differentiating effect on mammary epithelial cells and tumor suppressing activity against breast cancer. In this regard, we suggest to keep the names of MDGI and MRG when referring their functions on mammary gland and use H-FABP and B-FABP when referring their well-accepted FABP family phylogenetic tree (12).

A-3. The roles of MRG/B-FABP on mammary gland differentiation and suppression of breast cancer. FABPs comprise a well-established family of **cytoplasmic** hydrophobic ligand binding proteins and are thought to be involved in lipid metabolism by binding and intracellular transport of long-chain fatty acids. However, from other studies on role for FABPs in cell signaling, growth inhibition and differentiation has also been implied (12,16-17). In particular, H-FABP and B-FABP are abundantly expressed in differentiated mammary gland. It has been suggested that in heart and brain, FABPs regulate the supply of fatty acids to the mitochondria for beta-oxidation (18-19). The mammary gland, however, is a highly **lipogenic** tissue and fatty acids are not likely to be a major fuel for its metabolism. Within the phylogenetic tree of FABPs, B-FABP and H-FABP belong

to a closely related subfamily of proteins that act as tumor suppressors for breast cancer (12). Therefore, MRG and MDGI could fulfill different functions in brain and heart compared with mammary gland.

MDGI/H-FABP protein was mainly detected in myocardium, skeletal and smooth muscle fibres, lipid, and steroid synthesizing cells adrenals, lactating mammary gland, and terminally differentiated epithelia of the respiratory, intestinal and urogenital tracts (20). Within the similar content, the expression of MRG was mainly detected in **brain, heart**, and **skeletal muscle**, which are in the postmitotic status (2). Abundant MRG protein expression was also detected in human lactating mammary epithelial cells by immunohistochemical staining (21). These results provide evidence that expression of MRG and MDGI are associated with an irreversibly **postmitotic and terminally differentiated** status of cells. It has been previously demonstrated that the expression of B-FABP (mouse MRG) is correlated with neuronal differentiation in many parts of the mouse central nervous system (22-23) and blocking antibody to B-FABP can block glial cell differentiation in mixed primary cell cultures prepared during the first postnatal week (22). In mammary epithelium, MRG also induces mammary differentiation (21). These include that (a) overexpression of MRG in human breast cancer cells induced differentiated cellular morphology and a significant increase in the production of lipid droplets and (b) treatment of mouse mammary gland in organ culture with MRGp resulted in a differentiated morphology and production of β-casein (Appendix 3). Therefore, it seems clear that a differentiation-associated function is a common property of these structurally related subfamily of FABPs. Being the members of FABP family, the most characterized biological functions for MRG/B-FABP are tumor suppressing activities against breast cancer and differentiating effect on mammary cells. These include:

1). The loss of B-FABP/MRG expression (2) and H-FABP/MDGI (24) is associated with breast cancer progression.

2). Both MRG (21) and MDGI (11,25) are highly expressed in the fully differentiated lactating mammary gland and induce mammary differentiation.

3). MRG and MDGI have been mapped at the chromosome 6q22-23 (12) and 1p35 (26) that harbor the putative tumor suppressor genes for breast cancer (27-28).

4). Both MRG and MDGI strongly suppress the growth of breast tumors (2,26).

B.   WORK ACCOMPLISHED

Specific Aim 1: Does MRG overexpression in MMTV/MRG transgenic mice induce a pregnancy/lactation-like mammary gland differentiation?

SA1-1. Does MRG induce functional differentiation in mammary epithelial cells? (FINISHED)
Our in vitro studies suggest a differentiation-associated function of MRG on breast epithelial cells. In the current study, we established MRG transgenic mouse under the promoter of mouse mammary tumor virus (MMTV) and investigated the role of MRG on mammary gland differentiation. Our data indicate that MRG is a mediator in the differentiation effect of pregnancy on breast epithelial cells.

Stimulation of β-casein expression.
To determine if the mammary epithelial cells were functionally as well as morphologically differentiated, the expression of the milk protein gene β-casein was analyzed by RT-PCR. Fig. 1 shows a representative MRG transgene and β-casein expression in four virgin control mice and four randomly picked fourth generation virgin transgenic mice from MM-H1 and MM-H2 lines. RT-PCR analysis revealed the expression of the transgene MRG and β-casein in all four transgenic mice (Fig. 1, lines 6-9). However, no detectable β-casein transit was observed in age-matched control virgin mice (Fig. 1, lines 1-3). As expected, expression of β-casein was detected in an 8-day pregnant of

normal mouse (Fig. 1, line 4). These results indicate that the mammary glands of the established MMTV/MRG transgenic lines MM-H1 and MM-H2 have functional expression of the transgene, which stimulates mammary gland differentiation by expression of β-casein.
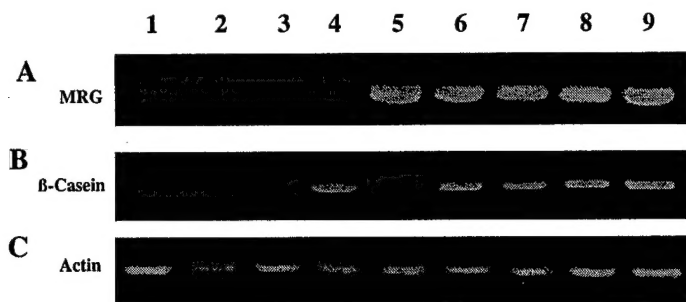
Fig. 1. RT-PCR analysis of MRG transgene and β-casein expression. Eight-week old fourth generation virgin MM-H1 and MM-H2 mice, and age matched control virgin mice and control pregnant mouse were sacrificed and the inguinal mammary glands were removed. Expression of MRG transgene (**A**) and β-casein mRNA (**B**) was analyzed by RT-PCR and normalized for β-actin expression (**C**). The 393-bp of the human MRG and the 480-bp of the mouse β-casein gene were amplified by PCR with sets of primer as described in Materials and Methods. *Lanes 1-4*, control mice; *lane 4*, control pregnant mouse; *lane 5*, T47D breast cancer cell was used as a positive control for MRG expression; *lane 6*, MM-H1 mouse; *lane 7*, MM-H1 mouse; *lane 8*, MM-H2 mouse, *lane 9*, MM-H2 mouse.

SA1-2. Does MRG induce morphological as well as functional differentiation? We will study if MRG is a local signal driving the pregnancy-like differentiated morphology with lobuloalveoli and epithelial branching and in MMTV/MRG transgenic mouse.

Effects of expression of MRG transgene on mammary gland development and differentiation

Because MRG protein expression was associated with human mammary gland differentiation with the highest expression observed in the differentiated alveolar mammary epithelial cells from the lactating gland, we were interested in studying whether MRG is an instigator of mammary gland differentiation or merely a correlative product during mammary gland development. The effect of transgene expression on mammary gland development and functional differentiation was assayed by morphological analyses of ductal elongation and appearance of a differentiated alveolar branching morphogenesis. While the mammary gland development starts at about 3-week old in wild-type mice with ductal elongation and development of the initial branching structure, the functional differentiation starts at the onset of pregnancy with the expansion of secretory lobulo-alveolar architect. Whole mount preparations of the mammary glands from virgin wild-type and virgin transgenic mice were examined to determine the effect of MRG on mammary gland development. Fig. 1 shows a representative mammary gland analysis of 32-day old transgenic mouse vs. wild-type control littermate. Mammary ducts in the transgenic virgin as well as in the control virgin littermate filled the typical ½ length of the inguinal gland and appeared normal (Fig. 2, compare A and B), indicating that expression of the transgene did not alter the ductal outgrowth during the early mammary gland development. However, an alternation in the developmental pattern of the distal cells of ducts in transgenic virgin mice (Fig. 2D) was observed
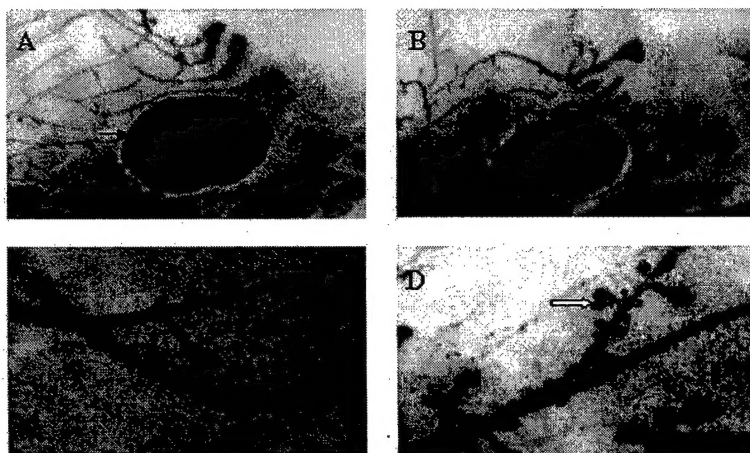
compared with the control littermate (Fig. 2C). While the limited budding was developed in the wild-type gland (Fig. 2C), transgenic gland exhibited multiplicity of budding (Fig. 2D).

Fig. 2. Whole mount histological analysis of mammary gland from female MM-H2 transgenic mouse and wild-type littermate. A 32-day old virgin MM-H2 mouse and a age-matched virgin wild-type littermate mouse were sacrificed, the right inguinal gland were removed and subjected to whole mount gland fix, defat, and staining. **A & C**, wild-type control mouse. **B & D**, MM-H2 transgenic mouse. **A & B**, lower magnification images from (Nikon, 2X10). Arrows indicate the inguinal lymph node and the direction for duct extension (from left to right). **C & D**, higher magnification pictures from (10X10). An open arrow indicates budding.

C.   KEY RESEARCH ACCOMPLISHMENTS

1. Expression of MRG transgene in the mammary gland resulted in differentiated gland morphology with increased formation of lobulo-alveoli.
2. Consistent with the morphological change, MRG stimulated milk protein β-casein expression in the gland of the transgenic mice.

D: CONCLUSIONS

The protective effect of pregnancy against breast cancer can be attributed to the transition from undifferentiated mammary epithelial cells in the nulliparous to differentiated mature cells during the pregnancy and lactation. The realization that specific reproductive endocrine events alter breast cancer risk in a predictable fashion raises the possibility that events known to decrease breast cancer risk might be mimicked pharmacologically. Unfortunately, the biological basis of parity-induced protection against breast cancer is unknown. A stumbling block in chemoprevention has been the prolonged and costly clinical trials required to determine the efficacy of chemoprevention regimens due to reliance on the development of breast cancer as a clinical end point. As such, the identification and use of intermediate **molecular end points** that accurately identify changes in the breast associated with parity would facilitate the development of such chemopreventive regimens. Within these contents, we have demonstrated that MRG, which are highly expressed in the differentiated pregnant mammary gland, induces the gland differentiation both morphologically and functionally. The potential application of MRG as a pregnancy-like differentiation factor for mammary gland and served as one of the intermediate molecular end points for chemoprevention warrant further investigation.

E. REFERENCES
1. H. Ji, Y.E. Liu, T. Jia, M. Wang, J. Liu, G. Xiao, B.K. Joseph, C. Rosen and <u>Y.E. Shi</u>. Identification of a breast cancer-specific gene, BCSG1, by direct differential complementary DNA sequencing. **Cancer Res.**, 57: 759-764, 1997.
2. <u>Y. Eric Shi</u>, Jian Ni, Guowei Xiao, Yiliang E. Liu, Alexander Fuchs, Guoliang Yu, Jeffery Su, John M, Cosgrove' Lily Xing, Mei Zhang, Jiyou Li, Bharat B. Aggarwal, Anthony Meager, and Reiner Gentz. Antitumor activity of the novel human breast cancer growth inhibitor MRG. **Cancer Res.**, 57 (15): 3084-3091, 1997.
3. Bielka H, Grosse R, Bohmer F, Junghahn I & Binase B. Inhibition of proliferation of Ehrlich ascites carcinoma cells is functionally correlated with reduced activity of the cytosol to stimulate protein synthesis. Biomed. Biochim. Acta., *45*: 441-445, 1986.
4. Böhmer FD, Mieth M, Reichmann G, Taube C, Grosse R & Hollenberg MD. A polypeptide growth inhibitor isolated from lactating bovine mammary gland (MDGI) is a lipid-carrying protein. J. Cell Biochem., *38*: 199-204 , 1988.

5. Unterberg C, Borchers T, Hojrup P, Roepstorff P, Knudsen J & Spener F. Cardiac fatty acid-binding proteins. Isolation and characterization of the mitochondrial fatty acid-binding protein and its structural relationship with the cytosolic isoforms. J. Biol. Chem., 265: 16255-16261, 1990.

6. Brandt R, Pepperle M, Otto A, Kraft R, Bohmer FD & Grosse R. A 13-KD protein purified from milk fat blobule membranes is closely related to a mammary derived growth inhibitor. Biochemistry , 27: 1420-1425, 1988.

7. Yang Y, Spitzer E, Kenney N, Zschiesche W, Li M, Kromminga A, Muller T, Spener F, Lezius A & Veerkamp JH. Members of the fatty acid binding protein family are differentiation factors for the mammary gland. J. Cell. Biology ,127: 1097-1108, 1994.

8. Kurta A, Vogel, Funa K, Heldin CH & Grosse R.. Developmental regulation of mammary-derived growth inhibitor expression in bovine mammary tissue. J.Cell Biol., 110 (5): 1779-1789, 1990.

9. Binas B, Spitzer E, Zschiesche W, Erdmann B, Kurtz A, Muller T, Niemann C, Blenau W & Grosser R. Hormonal induction of functional differentiation and mammary-derived growth inhibitor expression in cultured mouse mammary gland explants. In Vitro Cell Dev. Biol., 28A: 625-634, 1992.

10. Li M, Spitzer E, Zschiesche W, Binas B, Parczyk K & Grosse R. Antiprogestins inhibit growth and stimulate differentiation in the normal mammary gland. J. Cell Physiol., 164: 1-8, 1994.

11. Yang Y, Spitzer E, Kenney N, Zschiesche W, Li M, Kromminga A, Muller T, Spener F, Lezius A & Veerkamp JH. Members of the fatty acid binding protein family are differentiation factors for the mammary gland. J. Cell. Biology ,127: 1097-1108 , 1994.

12. Y. Eric Shi. Correspondence re: Y.E. Shi et al., Antitumor activity of the novel human breast cancer growth inhibitor, mammary-derived growth inhibitor-related gene, MRG. Cancer Res., 58: 4015-4017, 1998.

13. Böhmer FD, Mieth M, Reichmann G, Taube C, Grosse R & Hollenberg MD. A polypeptide growth inhibitor isolated from lactating bovine mammary gland (MDGI) is a lipid-carrying protein. J. Cell Biochem., 38: 199-204 , 1988.

14. Specht B, Bartetzko N, Hohoff C, Kuhl H, Franke R, Borchers T & Spener F. Mammary derived growth inhibitor is not a distinct protein but a mix of heart-type and adipocyte-type fatty acid-binding protein. J. Biol. Chem., 271: 19943-19949, 1996.

15. Treuner M, Kozak CA, Gallahan D, Grosse R & Muller T. Cloning and characterization of the mouse gene encoding mammary-derived growth inhibitor/heart-fatty acid-binding protein. Gene., 147(2): 237-242, 1994.

16. Kurtz A, Spitzer E, Zscuesche W, Wellstein A, and Grosse R. Local control of mammary gland differentiation: mammary derived growth inhibitor and pleiotrophin. Bioche. Soc. Symp. 63: 51-69, 1998.

17. Borchers T, Hohoff C, Buhlmann C, and Spener F. Heart-type fatty acid binding protein-involvement in growth inhibition and differentiation. Prostaglandins Leukot. Essent Fatty Acids, 57: 77-84, 1997.

18. Bass NM and Manning JA. Tissue expression of three structurally different fatty acid binding proteins from rat heart muscle, liver and intestine. Biochem. Biophys. Res. Commun. 137: 929-935, 1986.

19. Billich S, Wissel T, Kratzin H Hahn U, Hagenhoff B, Lezius AG and Spener F. Cloning of a full-length complementary DNA for fatty-acid-binding from boving heart. Eur. J. Biochem. 175: 549-556, 1988.

20. Zschiesche W, Kleine AH, Spitzer E, Veerkamp JH and Glatz JF. Histochemical localization of heart-type fatty-acid finding protein in human and murine tissues. Histochem. Cell. Biol., *103*: 147-156, 1995.

21. Mingsheng Wang, Yiliang E. Liu, Jian Ni, Banu Aygun, Itzhak D. Goldberg, Y. Eric Shi. Induction of mammary differentiation by MRG that interacts with ω-3 fatty acid on growth inhibition of breast cancer cells. ***Cancer Res***. 60: 6482-6487, 2000.

22. Feng L, Hatten ME and Heintz N. Brain lipid-binding protein (BLBP): a noval signaling system in the developing mammalian CNS. Neuron *12 (4)*: 895-908, 1994.

23. Anton ES, Marchionni MA, Lee KF and Rakic P. Role of GGF/neuregulin signaling in interactions between migrating neurons and radial glia in the developing cerebral cortex. Development *124 (18)*: 3501-10, 1997.

24. Huynh H, Alpert L and Pollak M. Silence of the mammary-derived growth inhibitor (MDGI) gene in breast neoplasms is associated with epigenetic changes. Cancer Res., 56: 4865-4870, 1996.

25. Kurta A, Vogel F, Funa K, Heldin CH & Grosse R. Developmental regulation of mammary-derived growth inhibitor expression in bovine mammary tissue. J.Cell Biol., *110 (5)*: 1779-1789, 1990.

26. Huynh H, Larsson C, Narod S and Pollak M. Tumor suppressor activity of the gene encoding mammary-derived growth inhibitor. Cancer Res., 55: 2225-2231, 1995.

27. Theile M, Seitz S, Arnold W, Jandrig B, Frege R, Schlag PM, Hansch W, Guski H, Winzer KJ, Barrett JC and Scherneck S. A defined chromosome 6q fragment (at D6S310) harbors a putative tumor suppressor gene for breast cancer. ncogene, *13*: 677-685, 1996.

28. Bieche I, Champeme MH & Lidereau R. A tumor suppressor gene on chromosome 1p32-pter controls the amplification of MYC family genes in breast cancer. Cancer Res., *54*: 4274-4276, 1994.

29. Xu LZ, Sanchez R, Sali A and Heintz N. Ligand specificity of brain lipid-binding protein. *J. Biol. Chem. 271 (40)*: 24711-24719, 1996.

30. Kaizer F, Boyd NF, Kriukov V, Trichler D: Fish consumption and breast cancer risk: an ecological study. *Nutr Cancer 12*: 61-68, 1989.